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Immunohistochemistry of Diverging and Converging Neurotransmitter Systems in Mollusks

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Abstract. This series of studies was undertaken to compare the distribution of several transmitter-specific neuron systems in the nervous systems of the marine mollusks, *Aplysia californica* and *Pleurobranchaea californica*. Several specimens of each of the major ganglia of both species were sectioned serially, and each series was stained immunohistochemically to reveal one of the neuron systems. The present paper reports the results of stainings for acetylcholine, histamine, serotonin, gamma-aminobutyric acid (GABA), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), Phe-Met-Arg-Phe-NH_2 (FMRFamide), and small cardioactive peptide B (SCP_B). For all the transmitter-specific sets of neurons examined, relatively few neurons send diverging projections to large areas of the neuropil in one or more ganglia. Moreover, different transmitters converge onto similar areas, and several transmitters evidently project onto the same identified neuron. Many of these diverging and converging projections are sufficiently extensive and overlapping that they are unlikely to be specific for a particular motor-pattern. The overall findings are consistent with our previous neurophysiological data. These indicate that activity does not necessarily arise through circuit-specific, identifiable connections. Instead, appropriate response patterns, often containing mixtures of several behaviors, emerge variably through diffuse connections. These findings are also consistent with recent reports from other laboratories indicating that even light topical stimulation generates highly distributed neural activity, and that the responses of identifiable neurons are not constant. The anatomical data presented here should be combined with physiological experiments aimed at verifying the neurotransmitter action of the substances examined and elucidating the role of these agents in controlling the dynamics of neuronal responses.

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Introduction

Adaptive systems, whether they be chemical, cellular, or organismal, are composed of many components that must communicate with one another if the systems are to generate coherent responses. The nervous system represents a special form of communication between cells characterized by the anatomical connection of the neurons with each other and with target cells. Communication occurs in two forms: electrical potentials are propagated along the neuronal membrane, and they are relayed to recipient cells through chemical neurotransmission. According to the classical view, nonspecific electric signals are prevented during conduction through mechanical isolation by glial cells, and chemical signalling is locally limited at the synapse through rapid breakdown of the transmitter substances. These arrangements allow individual neurons to be connected specifically with other neurons so that fixed circuits are formed. Many examples show that simple reflexes are produced by response-specific circuits. However, recent evidence suggests that even relatively simple reflexes, such as the *Aplysia* gill withdrawal reflex, may not necessarily be produced by stereotypically identified neurons (Leonard *et al.*, 1990). Furthermore, our analysis of molluscan feeding and other buccal-oral behaviors, and of the neuronal activity producing them, has suggested that such behaviors may not be based on behavior-specific circuits (Cohan, 1980; Cohan and Mpitsos, 1983a, b; Mpitsos and Cohan, 1986a, b; Mpitsos and Lukowiak, 1985).

More specifically, our findings on the *Pleurobranchaea* buccal and cerebral ganglia indicate that the neurons producing oral-buccal behaviors are multifunctional, so that

the same set of connections generates many different behaviors. Our physiological and behavioral studies have shown that many mouth-related behaviors of *Pleurobranchaea* arise from a matrix of highly interconnected neurons showing the following characteristics: (1) extensive divergence occurs by which a single neuron connects with many others; (2) convergence occurs by which many neurons innervate the same neuron; (3) neurons feed back to neurons that drive them; (4) motor neurons are coupled to one another; (5) and the most important feature may be that the nervous activity and the corresponding behaviors are variable, noisy and chaotic (Mpitsos and Cohan, 1986a; Mpitsos *et al.*, 1988a, b). Variability expresses itself, for example, through the ability of neurons to drastically change their temporal firing order [*e.g.*, Fig. 5 in (Mpitsos and Cohan, 1986b], or the ability of a characterized set of neurons to reorganize into coherent action after a critically important neuron that controls the patterned activity has been functionally removed from the circuit [*e.g.*, Fig. 16 B and C in (Mpitsos and Cohan, 1986b]. Our simulation studies indicate that even random noise may have a controlling role in the ability of animals to learn or to generate activity patterns that are appropriate for a given environmental situation (Burton and Mpitsos, 1991).

The basic issue, therefore, in such parallel, distributed systems is to understand the implications of self-organizing action of groups of neurons that emerges from the underlying converging and diverging connections, and the role of variability in such processes (Mpitsos, 1989; Burton and Mpitsos, 1991). The traditional way to define neuroanatomical connectivity has largely been one based on characterization of individual neurons. This method has been valuable in revealing hierarchically different forms of neuronal activity. But it is bound to be incomplete because it only accounts for contributions by the identified elements of the system. The possibility that the unidentified elements may or may not affect the system is, by definition, neglected. This omission is critical, for evidence is accumulating that even simple reflexes may involve a significant proportion, if not most, of the neurons in a ganglion. Studies of *Aplysia* gill withdrawal movements elicited by light tactile stimulation of the siphon skin in *Aplysia* show that 300 to 400 of the 1000 neurons present in the ganglion become active (Zečević *et al.*, 1989). Moreover, as has also been observed in *Pleurobranchaea*, different subsets of these neurons in *Aplysia* become active in response to successive stimulations (Wu *et al.*, 1989). By contrast, the identified neurons constitute only a small fraction of the total neuron population (Kandel, 1979), and detailed analyses of variability in their responses are presently lacking.

The variability in neuronal responsiveness is, in our view, controlled by a variety of mechanisms. One may operate through neurotransmitters by adjusting the bifurcation dynamics of the system, as shown in computer simulations of neuronal membranes (Chay, 1985). Another mechanism may be to control the effects of noise (Burton and Mpitsos, 1991).

An understanding of the transmitter-specific connectivity, therefore, provides a necessary foundation for elucidating both the emergence of cooperative activity in distributed neural networks and the generation and control of variability. Toward this end, we have mapped classical and putative neurotransmitter systems in the major ganglia of the sea slugs *Aplysia californica* and *Pleurobranchaea californica*. Special emphasis has been paid to the buccal and cerebral ganglia, because they contain the neurons responsible for feeding behavior, and many forms of feeding behavior have been correlated with the activity patterns of particular neurons (Mpitsos and Cohan, 1986b; Mpitsos and Lukowiak, 1985). Although several transmitters have been mapped in these ganglia, most work has been performed on whole-mounts and, to our knowledge, no systematic study on complete serial sections is available. Furthermore, these ganglia have not been mapped for such important transmitters as GABA and ACh. Also, since most previous mapping studies have dealt with one substance at a time, we have made an attempt to gather the pieces together to allow for comparisons. In the present paper we summarize the results obtained on eight transmitters or neuropeptides: acetylcholine, histamine, serotonin, gamma-aminobutyric acid (GABA), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), Phe-Met-Arg-Phe-NH_2 (FMRFamide), and small cardioactive peptide B (SCP_B).

In the experiments summarized here, we have asked whether the neurotransmitter-specific systems of each ganglion are distributed with high selectivity or whether they follow the same divergence and convergence strategies as those observed when these systems are examined neurophysiologically. For most substances studied, our observations are consistent with the latter possibility. Studies on invertebrates have demonstrated the neurobehavioral modulatory action of neurohumoral agents, and their ability to produce specific motor pattern changes (*e.g.*, Marder, 1984, 1988; Weiss *et al.*, 1986a, b; Dickenson and Marder, 1989; Rosen *et al.*, 1989; Dickenson *et al.*, 1990). The extensiveness of the divergence and convergence in our experimental systems favors the notion that the neurotransmitter systems collectively form another dimension of neurointegration, in addition to the neurophysiological variables. Many transmitters may act simultaneously, and the effect of a given substance depends on the context of all others present. This principle has been demonstrated recently for three interacting neuropeptides in the rat hypothalamus (Albers *et al.*, 1991). Much as behavior emerges from the melange of impinging

sensations in whole animals, so it may be that the effect of interacting neurohumors may emerge nonlinearly from the aggregate dynamics rather than through the effects arising from the experimentally enforced action of single agents.

Materials and Methods

Experimental animals

The marine mollusks *Aplysia californica* and *Pleurobranchaea californica* were obtained from Dr. R. Fay (Pacific Biomarine, Venice, California) and maintained in an open seawater system held at 15°C for *Aplysia* and 11°C for *Pleurobranchaea*. The animals were anaesthetized by injecting approximately ⅓ of body weight 0.3 *M* $MgCl_2$ into the body cavity. The nervous system was dissected in seawater, the nerve roots were identified and the preparation was pinned out in a dish coated with Sylgard (Dow Corning).

Histological sections

One of the following fixatives was used. For serotonin, CCK, and VIP: Bouin's solution overnight at 4°C. For histamine and GABA: 4% carbodiimide for 1 h at room temperature, followed by Bouin's solution overnight at 4°C. For FMRFamide and SCPB: 0.5% p-benzoquinone for 1 h at room temperature, followed by Bouin's solution overnight at 4°C. For acetylcholine: Bouin's solution was supplemented with 0.1% glutaraldehyde overnight at 4°C. The specimens were rinsed and transferred into phosphate-buffered saline (PBS), pH 7.6 containing 20% sucrose. The ganglion was placed on a horizontal freezing plate so that the dorsal surface (caudal surface for *Aplysia* buccal ganglion) showed upwards and the left-right orientation corresponded to that *in situ*. The plane of sectioning was adjusted parallel to the freezing surface. Cryostat sections (16 μm) were cut and placed on chrome-alum-gelatin-coated glass slides and rinsed in PBS containing 0.25% Triton X-100. The slides were incubated with 10% normal swine serum for 20 min at room temperature, then with the primary antiserum diluted in PBS-Triton for 24 to 48 h at 4°C, and finally with fluorescein- (FITC)- or rhodamine-(TRITC)-labeled swine immunoglobulins specific for rabbit IgG (DAKO F205 and R156) diluted 1:100 in PBS-Triton for 1 h at room temperature. All incubations were performed in a humid chamber. After rinsing in PBS, the sections were covered with glycerol-PBS (50/50) and examined and photographed with a Zeiss Universal microscope incorporating an HBO mercury lamp and filter blocks 487712 for TRITC (excitation maximum 546 nm, emission barrier > 590 nm) and 487709 for FITC (excitation maximum 450–490 nm, emission barrier > 520 nm).

Antisera

Polyclonal histamine antiserum was obtained from Dr. P. Panula (Department of Anatomy, University of Helsinki, Finland). Its specificity has been characterized in a previous report (Panula *et al.*, 1988), and it has been shown to stain the previously reported histaminergic cell in both species studied (Soinila *et al.*, 1990). It was used at a dilution of 1:10,000. Immunoreactivity was completely inhibited by preabsorption with 1 μg/ml solution of histamine-protein conjugate.

Polyclonal serotonin antiserum was raised in rabbits against serotonin-albumin conjugates according to the method of Steinbusch *et al.* (1978). It was used in dilution 1:1,000 and it stained identifiable serotonergic neurons in molluscan ganglia and in rat sympathetic ganglia, but failed to stain cells containing catecholamines or histamine (Rathouz and Kirk, 1988, Soinila *et al.*, 1990). Immunoreactivity was completely inhibited by preabsorption with 10 μg/ml solution of serotonin-protein conjugate.

Polyclonal acetylcholine antiserum was raised in rabbits against choline-hemocyanin conjugates. The antiserum was used in dilution 1:500, and it stained previously identified cholinergic neurons in *Aplysia* ganglia and in rat brain and spinal cord. Immunoreactivity was inhibited by preabsorption of the antiserum with 1 μg/ml solution of choline-albumin conjugate or with 10 m*M* acetylcholine. Specificity was also checked by incubation of consecutive sections with antiserum raised against glutarylated hemocyanin, which did not stain any neurons indicating that the staining obtained with acetylcholine antiserum was not due to immunoglobulins directed against the carrier protein. Further indication of specificity was obtained from dot blotting experiments in which the antiserum readily recognized the choline-protein conjugate but not other related conjugates.

Polyclonal GABA antiserum was raised in rabbits against GABA-hemocyanin conjugates. It was used in dilution 1:500, and it stained known GABAergic structures in the rat cerebellum. Specificity was confirmed in dot blotting experiments in which the antiserum recognized GABA-protein conjugate but not the conjugates containing glutamate, glutamine, aspartate, or beta-alanine. Immunoreactivity was completely inhibited by preabsorption with 1 μg/ml GABA-hemocyanin solution.

Polyclonal antisera against VIP, FMRFamide, and CCK were purchased from Incstar (Stillwater, Minnesota), and they were used in dilution 1:250–1:500 for VIP, and 1:500 for FMRFamide and CCK. Specificity of these antisera has been characterized by the manufacturer.

Monoclonal antiserum against SCPB was obtained from the Monoclonal Laboratory, Department of Zoology, University of Washington (Seattle, WA) and it has been characterized in a previous study (Kempf *et al.*, 1987).

Electrophysiological identification of neurons

The abdominal ganglion was bathed in artificial seawater (Instant Ocean from Aquarium Systems, Inc.) containing 0.3 *M* sucrose, and its surface was desheathed mechanically. Neuron R15 was located in the right dorsal-caudal region and neuron L10 in the left ventral-caudal region, based on previously established maps (Kandel, 1979). Electrophysiological criteria of identification was confirmed by intracellular recordings using standard electrophysiological methods and micropipettes filled with potassium chloride (0.8 *M*) or Lucifer Yellow (Sigma; at 50% saturated solutions at 4°C). Physiological recordings were made in artificial seawater solutions maintained at 12°C. Following intracellular recording, some cells were iontophoretically injected with Lucifer Yellow by passing hyperpolarizing current from the recording micropipette for at least 2 h before being fixed and processed for serial sectioning and immunohistochemistry. Examination of several preparations under bright light optics indicated that R15 can be reliably identified based on morphological criteria alone, as ascertained by examination of the same sections under UV-light, which makes the Lucifer Yellow-injected neurons visible.

Cobalt back-injection technique

The cerebral or the buccal ganglion and approximately 5 mm stump of the cerebral-buccal connective were dissected out into seawater, and the connective was mechanically desheathed. The ganglion was pinned out on a Sylgard-coated dish and the distal end of the connective was immersed in an isolated compartment of the dish that contained 0.5 *M* cobalt chloride in seawater. After incubation for 12 h at 4°C, the preparation was rinsed in seawater and immersed in 1% ammonium sulfide for 15 min or until black cobalt precipitate appeared. The preparation was dehydrated and cleared in alcohol-xylene series.

Mapping of transmitter-specific neuron systems

Complete serial cryostat sections through each ganglion were obtained for immunostaining. Due to the large number of sections (70–120 per ganglion), the distribution of stained cells in individual sections was reconstructed on diagrams representing consecutive zones. Definition of the zones was based on identified structures in serially sectioned ganglia stained with Cresyl fast violet.

Buccal ganglion; three zones. In *Aplysia,* the ganglion is located on the caudal, sometimes the ventrocaudal, aspect of the buccal mass; in *Pleurobranchaea,* the ganglion is consistently on the dorsal surface of the buccal mass. The plane of sectioning was adjusted parallel to the buccal mass, so that the denotation caudal-rostral in *Aplysia* buccal ganglion corresponds to dorsal-ventral in the *Pleurobranchaea* buccal ganglion. For reconstruction, the ganglion was divided into three zones: the coelomic zone contains the sections from the caudal (*Aplysia*) or dorsal (*Pleurobranchaea*) surface to the level where the commissure first appears; the commissural zone consists of the sections showing the commissure; and the buccal zone consists of the rest of the ganglion.

Cerebral ganglion; four zones. The dorsal zone extends from the ganglion surface to the level where the neuropil first appears. The dorsal subsurface zone extends to the dorsal edge of the anterior (*Aplysia*) or the larger (*Pleurobranchaea*) tentacular nerve. The commissural zone contains all sections showing the commissure, and the sections ventral to that level form the ventral zone.

Abdominal ganglion; four zones. The dorsal zone extends from the surface to the level where the neuropil first appears. The dorsal and ventral subsurface zones contain the sections showing the commissure, divided equally between the two zones. The sections ventral to the commissure constitute the ventral zone. The *Pleurobranchaea* abdominal (visceral) ganglion is tiny and could not be subjected to the above division.

Pedal-pleural ganglion; three zones. The dorsal and ventral zones extend from the respective surfaces to the level of the neuropil, and the central zone contains the rest of the ganglion.

Results

Mapping of transmitters in Aplysia californica

Buccal ganglion. Histamine immunoreactivity was confined to a pair of 100 μm neurons located ventrally in the commissural zone in each hemiganglion and to a very dense plexus throughout the neuropil (Figs. 1A–2A). This plexus contained both fine varicose terminals and coarser nerve fibers which were confined to the neuropil region.

No *serotonin*-immunoreactive neurons were seen in the buccal ganglion, although the neuropil showed a dense serotonin-immunoreactive plexus throughout (Figs. 1B, 2B). Serotonin-immunoreactive varicose fibers were very thin and were closely associated with the somas of most buccal neurons.

Each hemiganglion contained eight *acetylcholine*-immunoreactive neurons, two on the coelomic (caudal) surface, three in the commissural zone, and three on the buccal (rostral) surface (Fig. 1C). Acetylcholine-immunoreactive nerve terminals formed a dense plexus, evenly distributed throughout the neuropil. A large proportion of buccal neurons showed Ach-immunoreactive varicosities around their soma (Fig. 2C).

GABA-immunoreactive neurons consisted of a paired cluster on the caudal surface containing less than 10 re-

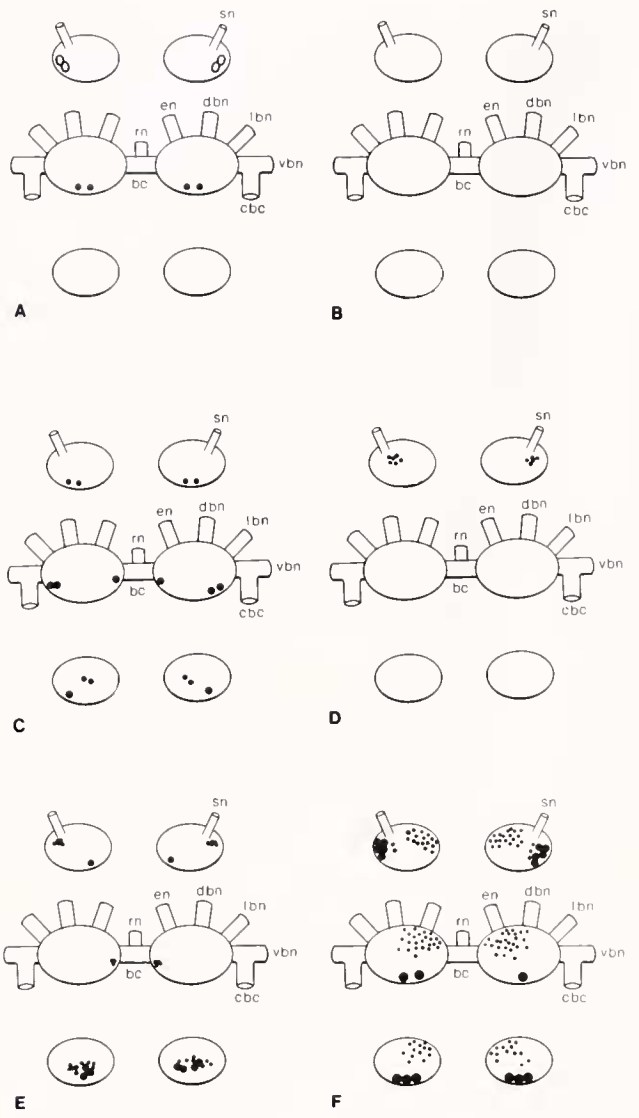


Figure 1. Schematic presentation of immunohistochemically identified neurons (black circles) in *Aplysia* buccal ganglion showing immunoreactivities for histamine (A), serotonin (B), acetylcholine (C), GABA (D), VIP (E), and FMRFamide (F). Open circles in A indicate the location of neurons B1 (lateral) and B2 (medial). Each set of three drawings summarizes the observations made in complete series through the ganglion. The top drawing represents the most caudal zone and the bottom drawing the most rostral zone. bc = buccal commissure, cbc = cerebral-buccal connective, dbn = dorsal buccal nerve, en = esophageal nerve, lbn = lateral buccal nerve, rn = radular nerve, sn = salivary nerve, vbn = ventral buccal nerve.

active cells per cluster (Fig. 1D). The GABA-immunoreactive neuropil consisted of fine, varicose fibers and terminals, and it was distributed throughout the neuropil region (Fig. 2D).

Each hemiganglion contained approximately 30 *VIP*-immunoreactive neurons (Fig. 1E). A single 100 μm cell and a cluster of small cells were located on the coelomic (caudal) surface. In the commissural zone, a group of a few small cells was located just ventral to the commissure. A larger group of VIP-immunoreactive cells was located on the buccal (rostral) surface. A VIP-immunoreactive fiber plexus was seen throughout the neuropil (Fig. 2E).

CCK-immunoreactivity was seen in 10–15 cells, most of which were located on the dorsal edge of the coelomic (caudal) surface. Two to three small cells were found on the rostral surface. A CCK-immunoreactive plexus was present throughout the neuropil (data not shown).

FMRFamide immunoreactivity was observed in a large number of cells throughout the ganglion, including most small neurons of the S-cluster and such identified neurons as B1 and B2 (Fig. 1F). FMRFamide-immunoreactive fibers formed a very dense plexus in all parts of the neuropil (Fig. 2F). This plexus was similar in appearance to, although considerably denser than, the histamine-immunoreactive plexus.

SCP_B-immunoreactive neurons were fewer in number than those showing reactivity for FMRFamide. Notably, neurons B1 and B2 were immunopositive. A dense SCP_B-immunoreactive plexus extended throughout the neuropil (data not shown).

Cerebral ganglion. A total of 30–40 *histamine*-immunoreactive neurons were found in each hemiganglion. They consisted of a small cluster in the dorsal subsurface zone, a pair of 100 μm neurons and a cluster of small neurons in the frontal region, a large cluster including the neuron C2 in the lateral region in the commissural zone, and another large cluster on the ventral surface (Fig. 3A). Histamine-immunoreactive varicose fibers were distributed throughout the neuropil region.

Serotonin-immunoreactive neurons consisted of 20–30 cells in each hemiganglion (Fig. 3B). These include the neuron C1 and a cluster of smaller cells around it. Additional serotonin-immunoreactive neurons consisted of a large cluster in the central region of the dorsal surface and of 4–5 100 μm neurons in the posterior lobes. Serotonin-immunoreactive fibers were found in all parts of the neuropil.

Approximately 20 *acetylcholine*-immunoreactive neurons were found in each hemiganglion (Fig. 3C). They consisted mainly of two compact clusters: one in the lateral region, and the other in the rostral-medial region of the commissural zone. In addition, a few single cells were located on the ventral surface. The acetylcholine-immunoreactive fiber plexus was sparse, and it was located close to the two compact clusters.

The cerebral ganglion contained 15–20 *GABA*-immunoreactive neurons in each hemiganglion (Fig. 3D). These cells were small and formed two clusters, one in the lateral region and the other in the rostral region. The GABA-immunoreactive neuropil formed a dense plexus that extended from the lateral region around the neuron C2, to the frontal GABA-immunoreactive cluster and

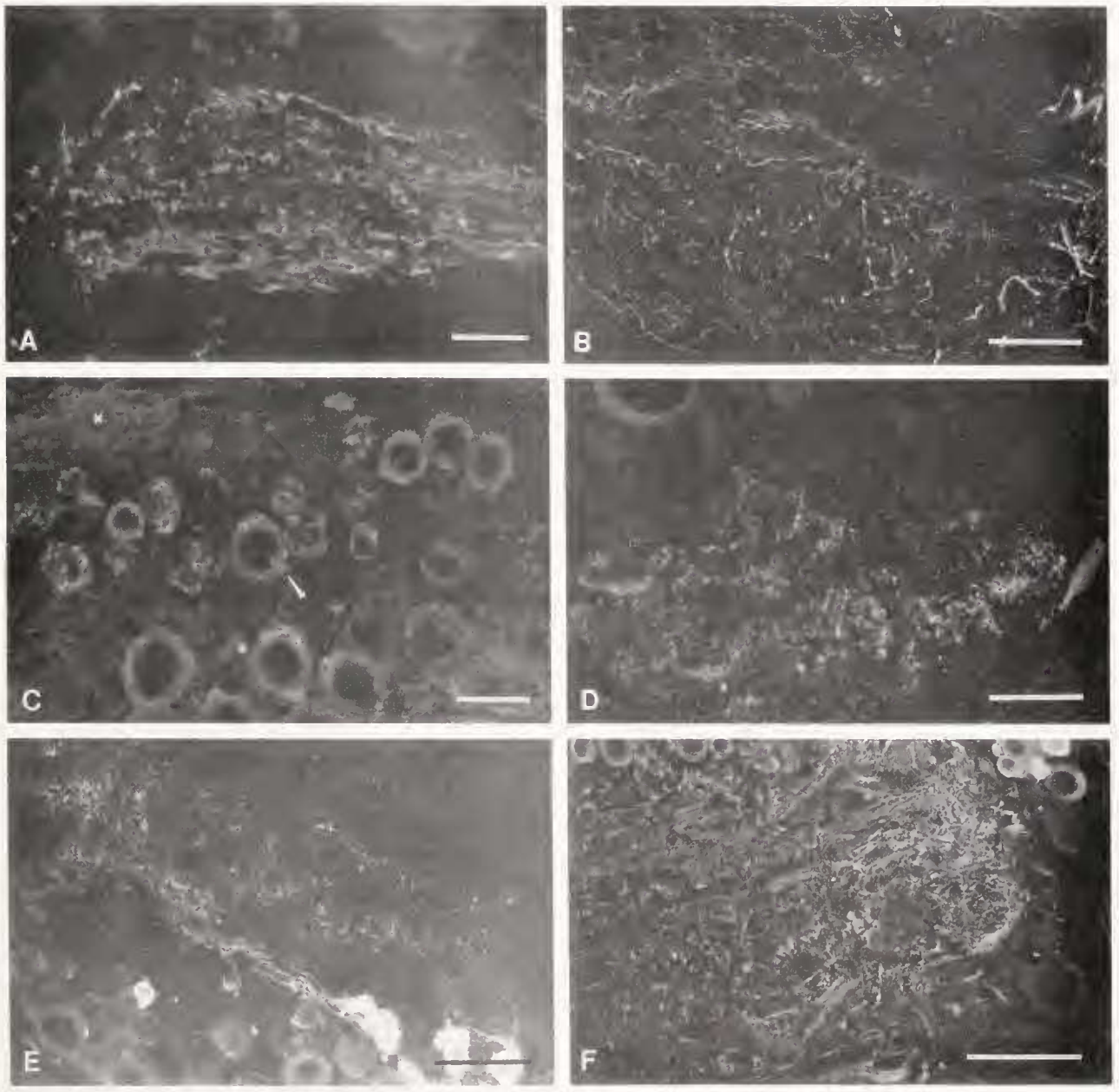
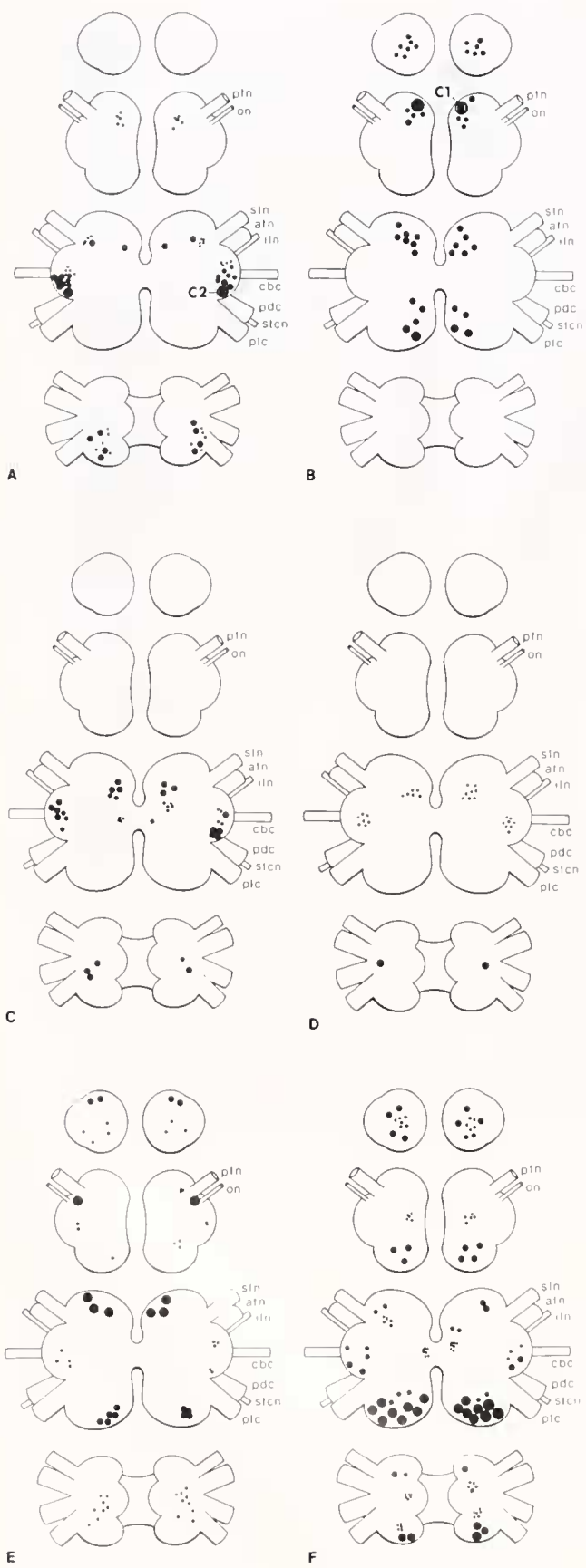


Figure 2. Photomicrographs of the neuropil region of *Aplysia* buccal ganglion showing immunoreactivity for histamine (A), serotonin (B), acetylcholine (C), GABA (D), VIP (E), and FMRFamide (F). Cross in C indicates immunoreactive neuropil and the arrowhead shows immunoreactive terminals around nonreactive neurons. Bar = 100 μm (A, D, E, F) or 50 μm (B, C).

further through the commissure to the same regions of the contralateral side. The other regions of the neuropil contained only few GABA-immunoreactive fibers.

Twenty to thirty neurons in each hemiganglion showed *VIP*-immunoreactivity (Fig. 3E): two large (200 μm) cells on the anterior edge, and some smaller solitary cells were found on the dorsal surface. The dorsal subsurface zone contained a large cell lateral to the outlet of the superior labial nerve and a few small cells on the medial and lateral edges. In the commissural zone, three large neurons were found in the anterior region, and small cells were scattered in the inner neuron rind. A cluster of four to seven neurons was located in the posterior region. The ventral surface contained several small VIP-reactive cells. A dense network of VIP-immunoreactive fibers was seen throughout the neuropil.

About 50 neurons showed immunoreactivity for *CCK*. Three large CCK-immunoreactive neurons were located



along the dorsal edge of the ganglion. The other cells were small neurons along the inner neuronal rind. A dense CCK-immunoreactive plexus was present throughout the neuropil region (data not shown).

A large number of neurons showed immunoreactivity for *FMRFamide*. These cells were found in all parts of the ganglion and were especially numerous in the posterior region where several large neurons stained (Fig. 3F). The neuropil showed a dense FMRFamide-immunoreactive plexus.

Fewer neurons were immunoreactive for SCP_B than for FMRFamide, but the staining revealed a dense plexus in all parts of the neuropil.

Abdominal ganglion. Histamine-immunoreactive neurons were found in three small clusters: one each on the lateral right and left margins of the dorsal subsurface zone, and a third one on the posterior edge of the ventral subsurface zone (Fig. 4A). A plexus of histamine-immunoreactive fibers was seen throughout the neuropil.

Serotonin-immunoreactivity was localized in several small neurons throughout the ganglion, but none of the large identified neurons showed immunoreactivity (Fig. 4B). All large neurons, including R2, R14, R15, L2-L7, and L10, received serotonin-immunoreactive innervation on their soma (Fig. 5A). Serotonin-immunoreactive fibers were diffusely distributed all over the neuropil region.

Of the large identified neurons, only L10 showed *acetylcholine* immunoreactivity; R2 was nonreactive (Fig. 4C). A cluster of relatively large (150 μm) neurons in the right caudal quadrant and several small neurons throughout the ganglion showed intense acetylcholine immunoreactivity. The neuropil showed an evenly distributed network of acetylcholine-immunoreactive fibers.

No *GABA*-immunoreactive neurons were seen in the abdominal ganglion, although the neuropil contained an extensive, widely distributed GABA-immunoreactive plexus (Fig. 4D).

Each hemiganglion contained 7–8 large *VIP*-immunoreactive neurons and four clusters of small cells (Fig. 4E). A VIP-immunoreactive plexus was found throughout the neuropil.

Several large *FMRFamide*-immunoreactive neurons were identified in the serial sections, such as R2, L2–L5

Figure 3. Schematic presentation of immunohistochemically identified neurons (black circles) in *Aplysia* cerebral ganglion showing immunoreactivities for histamine (A), serotonin (B), acetylcholine (C), GABA (D), VIP (E) and FMRFamide (F). Each set of four drawings summarizes the observations made of complete series through the ganglion. The top drawing represents the most dorsal zone, and the bottom drawing the most ventral zone. atn = anterior tentacular nerve, cbc = cerebral-buccal connective, iln = inferior labial nerve, on = optic nerve, pdc = cerebral-pedal connective, plc = cerebral-pleural connective, ptn = posterior tentacular nerve, sln = superior labial nerve, stcn = statocyst nerve.

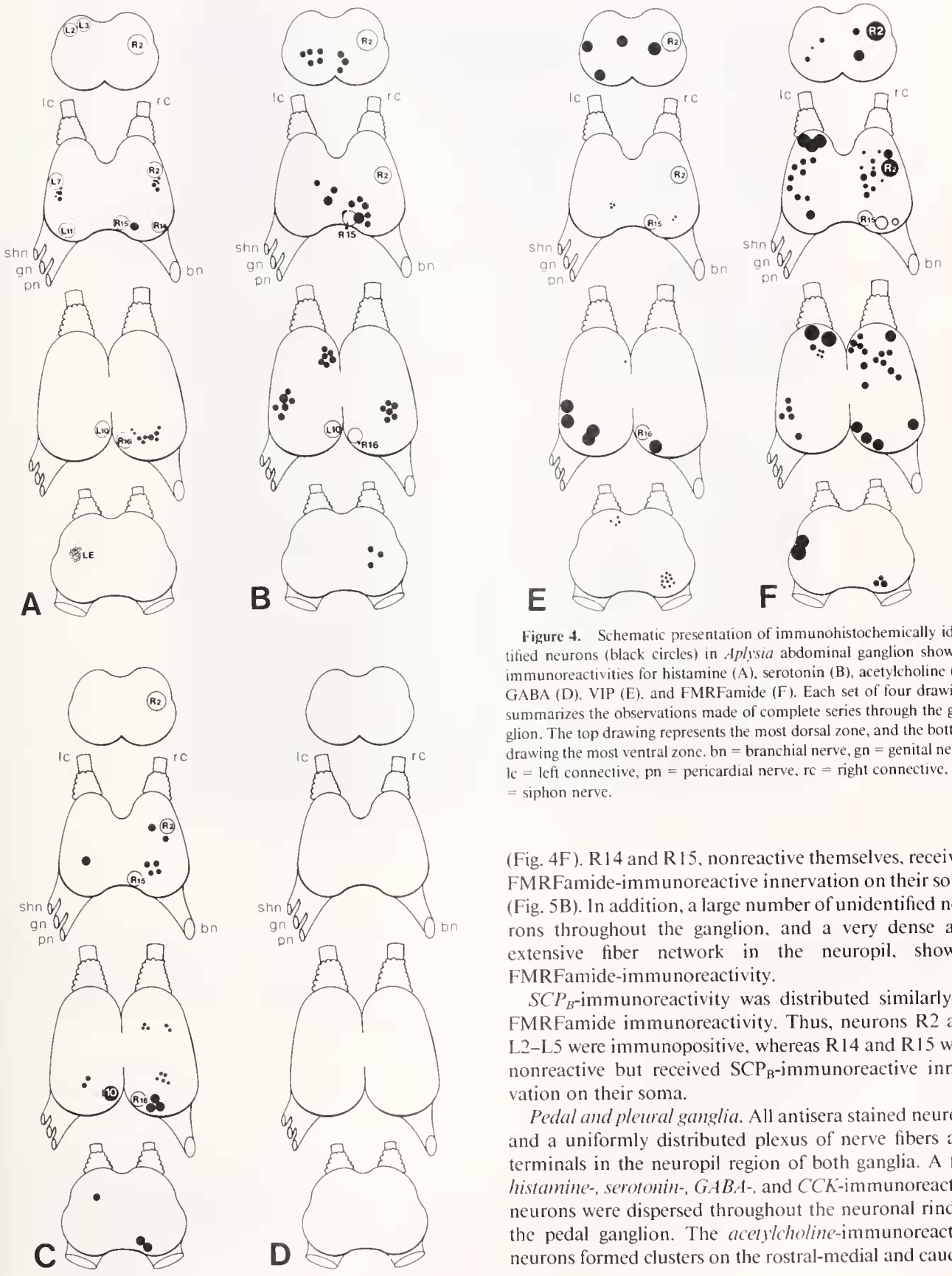


Figure 4. Schematic presentation of immunohistochemically identified neurons (black circles) in *Aplysia* abdominal ganglion showing immunoreactivities for histamine (A), serotonin (B), acetylcholine (C), GABA (D), VIP (E), and FMRFamide (F). Each set of four drawings summarizes the observations made of complete series through the ganglion. The top drawing represents the most dorsal zone, and the bottom drawing the most ventral zone. bn = branchial nerve, gn = genital nerve, lc = left connective, pn = pericardial nerve, rc = right connective, shn = siphon nerve.

(Fig. 4F). R14 and R15, nonreactive themselves, received FMRFamide-immunoreactive innervation on their soma (Fig. 5B). In addition, a large number of unidentified neurons throughout the ganglion, and a very dense and extensive fiber network in the neuropil, showed FMRFamide-immunoreactivity.

SCP_B-immunoreactivity was distributed similarly to FMRFamide immunoreactivity. Thus, neurons R2 and L2–L5 were immunopositive, whereas R14 and R15 were nonreactive but received SCP_B-immunoreactive innervation on their soma.

Pedal and pleural ganglia. All antisera stained neurons and a uniformly distributed plexus of nerve fibers and terminals in the neuropil region of both ganglia. A few *histamine-*, *serotonin-*, *GABA-*, and *CCK*-immunoreactive neurons were dispersed throughout the neuronal rind of the pedal ganglion. The *acetylcholine*-immunoreactive neurons formed clusters on the rostral-medial and caudal-

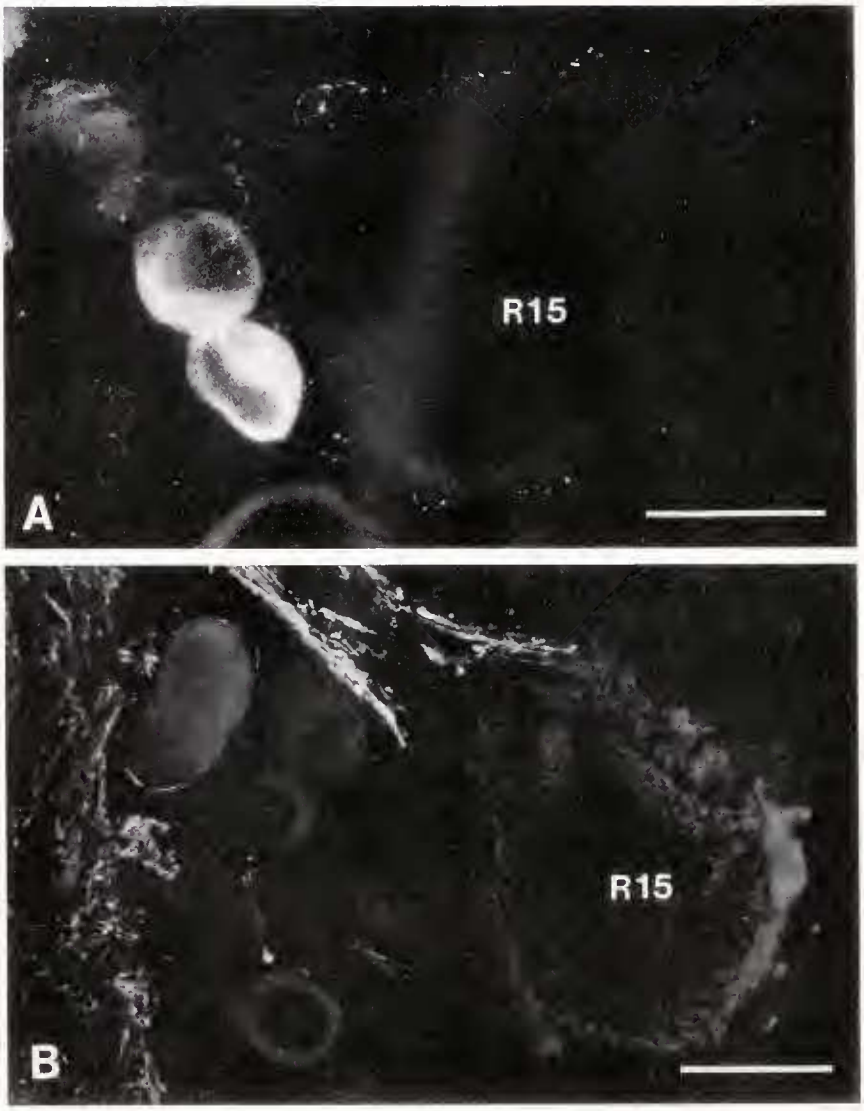


Figure 5. Neuron R15 of *Aplysia* abdominal ganglion. Serotonin- (A) and FMRFamide- (B) immunoreactive terminals surround intimately R15 soma (A) or its axon hillock region (B). Bar = 100 μm.

medial corners and on the caudal edge. The *VIP*-immunoreactive neurons were located mainly on the dorsomedial region. The number of FMRFamide- and SCP_B-immunoreactive neurons was greater than those for other stainings.

Back-injection of Aplysia *cerebral-buccal connective.* To examine the origin of the serotonin-containing innervation of the buccal neurons, the cerebral-buccal connective (CBC) was back-injected with cobalt chloride. Precipitation of cobalt-containing neurons in the cerebral ganglion revealed all cells that project into the CBC (Fig. 6). Distribution of the serotonin-immunoreactive neurons was obtained from serial sections (Fig. 3B). Comparison of the neuron populations revealed by the two techniques indicated that only one cell, the metacerebral giant cell (C1), stains with both methods. Serotonin immunoreactivity in cross-sections of the CBC was found in only one large axon profile. No serotonin-immunoreactive neurons were observed in the target tissues of the buccal ganglion, the esophagus, salivary gland, oral cavity mucosa, or the buccal muscles.

Mapping of neurotransmitters in Pleurobranchaea californica

Buccal ganglion. As in *Aplysia,* two large neurons on the dorsal surface showed *histamine* immunoreactivity. In addition, a cluster of about 10 small cells in each hemiganglion stained for histamine. All regions of the neuropil contained a very dense histamine-immunoreactive plexus

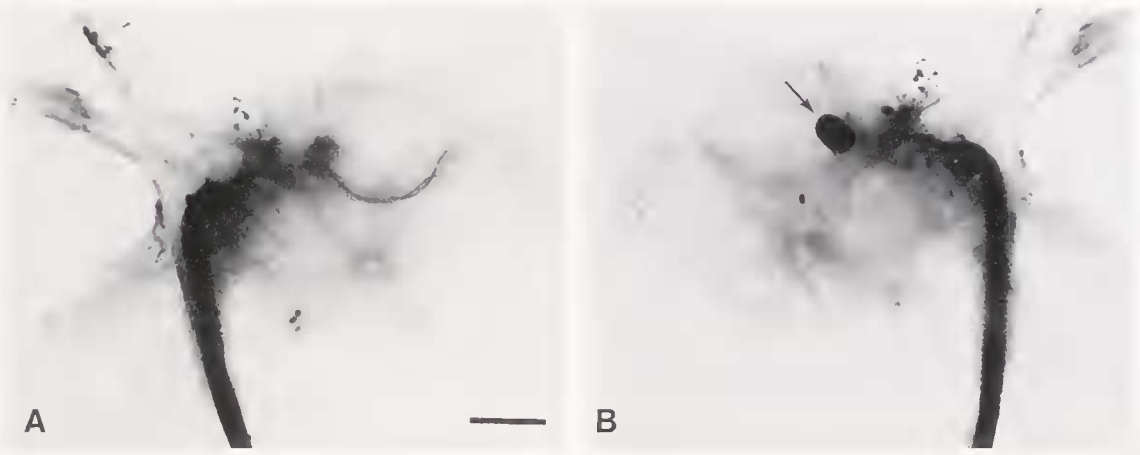


Figure 6. Whole *Aplysia* cerebral ganglion back-injected with cobalt chloride through the cerebral-buccal connective (black nerve trunk). A = ventral side, B = dorsal side. Arrow indicates neuron C1. Bar = 1 mm.

(Fig. 7A). In contrast to *Aplysia*, no *acetylcholine*-immunoreactivity was observed in this ganglion. A paired cell on the central coelomic (dorsal) surface and a cluster of small neurons on the buccal (ventral) surface showed immunoreactivity for *GABA* antiserum. A sparse GABA-immunoreactive plexus was present throughout the neuropil (Fig. 7B). Only two small clusters of *VIP*-immunoreactive cells were found in each hemiganglion. The ventral VIP-reactive cluster found in *Aplysia* was not present in *Pleurobranchaea*. VIP-immunoreactive plexus was present in the neuropil region. Several large (200–300 μm) *FMRFamide*- and *SCP*$_B$-immunoreactive neurons, including the neuron B1, were seen on the dorsal surface and on the posterior margin of the ganglion. The ventral surface contained a few immunoreactive neurons of varying size. Dense FMRFamide- and SCP_B-immunoreactive plexuses were present in all parts of the neuropil region (Fig. 7C).

Cerebral ganglion

Approximately 20 *histamine*-immunoreactive neurons were found in this ganglion, and they were distributed in all four zones. Histamine-immunoreactive fibers were seen in all parts of the neuropil. A few small *acetylcholine*-immunoreactive neurons were seen on the frontal lateral region of the dorsal surface. A distinct acetylcholine-immunoreactive plexus was present in a limited area close to the immunoreactive cells. Several small *GABA*-immunoreactive neurons were seen in the dorsal subsurface, commissural, and ventral surface zones located in a relatively narrow sector in the frontal part of the ganglion. A diffusely distributed GABA-immunoreactive fiber plexus was seen in the neuropil. Three clusters of small neurons, a pair of 50 μm neurons and a plexus in the neuropil showed *VIP* immunoreactivity. Many large (300 μm) and numerous smaller neurons scattered throughout the dorsal surface showed *FMRFamide*- and *SCPB*-immunoreactivity. On the ventral surface, several large neurons along the posterior margin, and two pairs of 150 μm cells in the frontal region, showed SCP_B immunoreactivity. The neuropil contained a very dense plexus of both FMRFamide- and SCPB-immunoreactive fibers.

Pedal-pleural ganglion complex. Staining with antisera against *histamine, serotonin, acetylcholine, GABA,* and *VIP* revealed a few immunoreactive neurons and an immunoreactive plexus throughout the neuropil. *FMRFamide* antiserum stained a large number of neurons throughout the ganglion, whereas the SCP_B antiserum stained only two clusters. Both FMRFamide and SCP_B antisera revealed a dense fiber plexus throughout the neuropil region.

Discussion

We report on the distribution of established neurotransmitters (histamine, serotonin, acetylcholine, GABA) and neuropeptides that are considered as putative neurotransmitters or -modulators (VIP, CCK, FMRFamide, SCP_B) in the nervous systems of *Aplysia* and *Pleurobranchaea*. We conclude, based on comparison of stained sets of cell bodies showing only little overlap, that the immunochemically identified neuronal populations represent largely separate pools. This conclusion is also supported by the observation that staining of the neuropil often exhibited a different pattern for different substances. Although examples of coexistence of a transmitter and neuropeptide have been described in individual *Aplysia* neurons (Brownstein *et al.*, 1974; Lloyd *et al.*, 1987; Ono, 1989; Cropper *et al.*, 1990), the present material indicates that there is no systematic colocalization of any two substances studied. With a few exceptions, we do not know

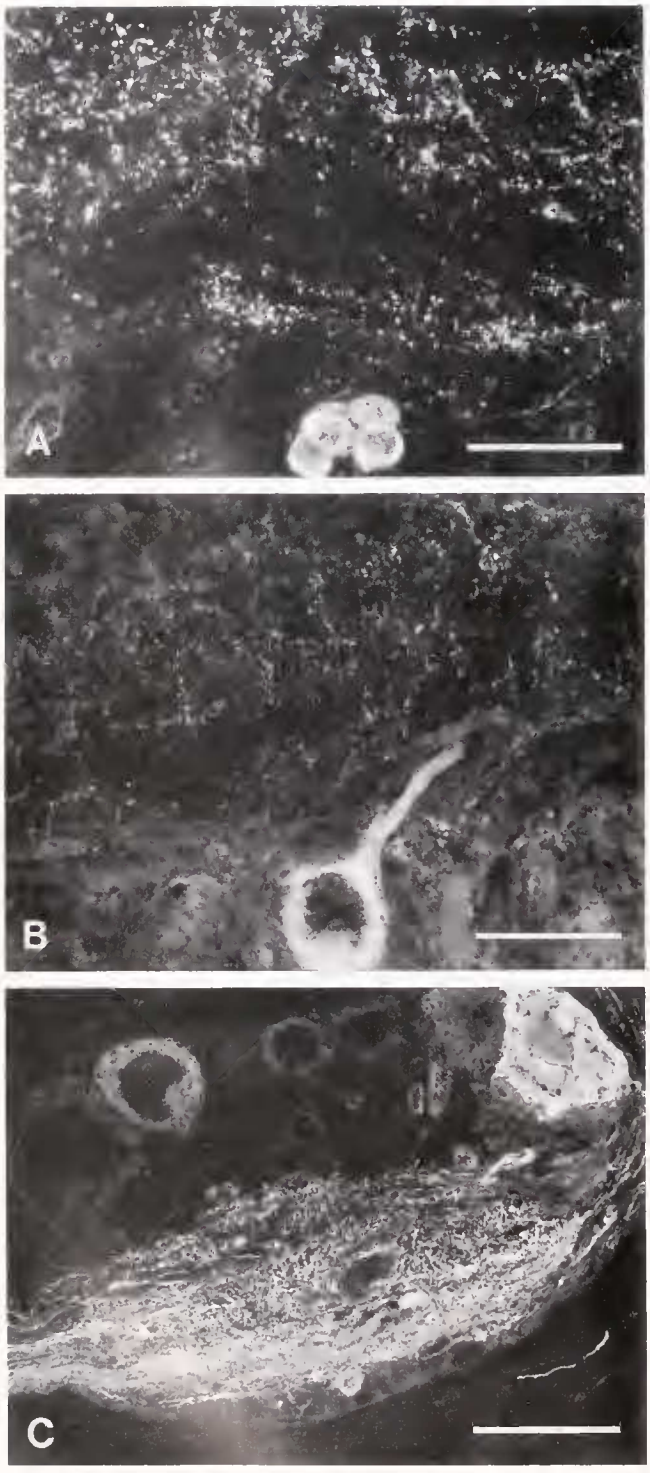


Figure 7. Photomicrographs of the neuropil region of *Pleurobranchaea* buccal ganglion showing immunoreactivity for histamine (A), GABA (B), and FMRFamide (C). Bar = 100 μm.

whether colocalization means corelease, or to what extent two colocalizing substances can be regulated independently. Consequently, the effect of transmitter colocalization on converging and diverging systems remains unknown.

Of the substances discussed here, histamine-, serotonin-, CCK-, FMRFamide- and SCP_B-immunoreactive neurons have been previously described in *Aplysia* ganglia (Ono and McCaman, 1984; Brown *et al.*, 1985; Kistler Jr. *et al.*, 1985; Longley and Longley, 1985; Ono, 1986; Lloyd *et al.*, 1987). In addition, identified molluscan neurons have been demonstrated to contain or use as transmitter histamine, acetylcholine, or GABA (*c.f.*, Leake and Walker, 1980). Our results, which are based on a complete series of sections, agree with the previous results largely obtained from whole ganglia. The use of serial sections has not only allowed us to study the distribution of stained nerve cell bodies, but also to examine transmitter-specific fiber systems both in the neuropil and around neuronal somata. Acetylcholine, which now has been localized immunohistochemically in a molluscan nervous system for the first time, is of particular importance in *Pleurobranchaea*, because the muscarinic cholinergic nervous system may be involved in associative learning in this species (Mpitsos *et al.*, 1988c).

Evaluation of the technique

The present study demonstrates that neurotransmitter systems in whole ganglia can be most effectively visualized and reconstructed through an examination of immunohistochemically stained, complete serial sections. In our study, reconstruction was performed manually and recorded on schematic drawings. For selected regions, this method can be supplemented by computer-assisted 3-dimensional analysis using commercially available programs. Serial sectioning offers the following advantages over whole-mount preparations: (1) uneven penetration of the reagents is largely avoided; (2) consecutive sections of the same ganglion can be used for double-staining or for controls with pre-immune serum; (3) better resolution of individual neurons and nerve terminals is obtained. In addition, the complete serial section technique allows regional analysis of the neuropil and visualization of somatic innervation of individual neurons, both features that have not been successfully revealed in whole-mounts. The main disadvantage of the current method is that it requires laborious reconstruction. Moreover, no single fixation method preserves the immunogenicity of all the substances studied, so staining of consecutive sections, each for a different transmitter, is often precluded. For example, histamine immunoreactivity is lost with formaldehyde fixation, serotonin immunoreactivity disappears even in low concentrations of glutaraldehyde, and peptide immunohistochemistry is superior in benzoquinone-fixed tissue. We have deliberately chosen the optimal fixation method for each substance. Another reason for staining

complete series of sections for each substance is that some transmitter-specific cells or cell clusters are very small and might easily be missed if the series is not complete.

Although specificity of immunoreactions was confirmed by preabsorption with synthetic antigens, or in the case of small molecules (histamine, serotonin, acetylcholine, GABA), with corresponding conjugates, the reservation must be considered that immunoreactivity does not necessarily represent the presence of the substance against which the antiserum was raised. For example, closely related peptides and their precursor molecules, or totally unknown tissue components may cause false interpretations. This problem is inherent in all immunohistochemical techniques, and the findings should be confirmed with pharmacological and chemical techniques. However, the observed immunoreactivities are essential for directing further physiological tests of the proposed hypothesis.

Comparison with previously reported transmitter maps

We have described in detail the distribution of histamine-immunoreactive neurons of *Aplysia* and *Pleurobranchaea* in a previous study (Soinila *et al.*, 1989, 1990) which has recently been confirmed by others (Elste *et al.*, 1990). Both studies failed to detect histamine immunoreactivity in L32 neurons of the *Aplysia* abdominal ganglion. Lack of histamine uptake by L32 neurons (Elste *et al.*, 1990) supports our immunohistochemical finding. These cells have been considered to be histaminergic because the responses to their intracellular stimulation are mimicked by exogenous histamine and blocked by specific histamine antagonist (Kretz *et al.*, 1986). In explaining this discrepancy, the possibility must be considered that the target cells on L32 neurons also receive histaminergic innervation from other sources, the blocking of which would in some way inhibit the response to stimulation of L32 cells.

Our maps of serotonin-immunoreactive neurons are in accordance with those published previously (Ono and McCaman, 1984; Kistler *et al.*, 1985; Longley and Longley, 1985).

No previous mapping studies on ACh immunoreactivity in *Aplysia* or *Pleurobranchaea* ganglia have been published, although several individual cholinergic neurons have been described in *Aplysia* (Giller and Schwartz, 1971; McCaman and Dewhurst, 1970; Gardner, 1971; Cohen *et al.*, 1978; Ono, 1989). The current evidence supporting the cholinergic nature of certain identified *Aplysia* neurons comes along two lines (1) Choline acetyl-transferase activity and conversion of exogenous choline into acetylcholine have been reported in microdissected B2, B4, B5, B7, B13, R2, L10, L11, LP1 (McCaman and Dewhurst, 1970; Giller and Schwartz, 1970; Eisenstadt *et al.*, 1973; Lloyd *et al.*, 1985). Except for B2, these cells also contain endogenous ACh (McCaman *et al.*, 1973; Ono, 1986). In addition, evidence has been presented that L10 releases acetylcholine from its terminals (Koike *et al.*, 1974). (2) Electrophysiological responses of the target cells of B4, B5, B15, B16, and B13 of the *Aplysia* buccal ganglion can be simulated by exogenous ACh and reversibly inhibited by cholinergic blockers (Gardner, 1971; Cohen *et al.*, 1978; Ono, 1989).

We consistently found eight pairs of ACh-immunoreactive neurons in the buccal ganglion. One of the ACh-immunoreactive cells on the coelomic (caudal) surface (Fig. 1) locates identically to B13 (Ono, 1989). The location of the cell close to the commissure correlates closely to that of B4 (Gardner, 1971). Two cells were found on the buccal (rostral) surface at locations that correlate closely with those reported for B15 and B16 (Cohen *et al.*, 1978). In contrast, B2 did not stain, and no ACh immunoreactive cells were found in the region of B5 or B7. ACh immunoreactivity was also observed in several cells in the cerebral ganglion (Fig. 3). No cholinergic neurons have been physiologically characterized in this ganglion, although it contains choline acetyltransferase at levels comparable to those found in the buccal and abdominal ganglia (McCaman and Dewhurst, 1970). We found several small cholinergic neurons in the *Aplysia* abdominal ganglion (Fig. 4C), but only one of the three previously reported large neurons claimed to be cholinergic stained with our antiserum. ACh immunoreactivity of L10 was confirmed by electrophysiological recording and subsequent Lucifer Yellow injection. We also found a relatively large neuron in the dorsal-caudal region, possibly L11. In contrast, R2, which was readily identified in sections without Lucifer Yellow injection, was consistently nonreactive. Likewise, the left pleural giant neuron LP1, a proposed homologue of R2, did not stain for ACh antiserum.

The following factors must be considered in explaining the discrepancies described above. (1) Although exogenous choline injected into the cell is converted into ACh by L10 and R2 (Koike *et al.*, 1972, 1974), under physiological conditions most ACh synthesis in these and other cholinergic cells may occur in the terminals. Indeed, the cell bodies of R2, L10, and L11 synthesize only minute amounts of ACh when incubated in the presence of excess exogenous choline (Eisenstadt *et al.*, 1973). (2) Contamination of microdissected neurons by nearby unidentified cholinergic cells may affect ACh measurements from single cells, as discussed previously by Giller and Schwartz (1971). In fact, we often found small ACh-immunoreactive cells close to R2 (Fig. 4C). (3) Simulation and blocking of the electrophysiological responses to stimulated, identified neurons as a sole method of transmitter identification must be interpreted with caution, as demonstrated

for L32 cluster (Elste *et al.*, 1990; Soinila *et al.*, 1990). Because systematic correlation of histochemistry to electrophysiology was not the aim of this study, the conclusion that, so far, only L10 identifies as cholinergic with chemical, histochemical, physiological, and pharmacological criteria is only preliminary and subject to extensive reinvestigation (Soinila and Mpitsos, in prep.). In conclusion, after confirming the specificity of our method by comparing the ACh staining with that produced by an antiserum against glutarylated carrier protein, we believe that the cells stained by our method are cholinergic, although the possibility cannot be excluded that the sensitivity of the method may limit visualization of neurons containing very low ACh concentrations. Notwithstanding this reservation, we consider the method useful for the purpose of the present study; *i.e.*, to map the distribution of ACh containing neurons and nerve fibers.

The distribution of GABA-immunoreactive neurons has not previously been studied in either *Aplysia* or *Pleurobranchaea*. Our findings, together with previous reports on the presence of GABA receptors in *Aplysia* neurons (Yarowsky and Carpenter, 1978; King and Carpenter, 1987), support the notion that GABA is a neurotransmitter in these species.

Although VIP-immunoreactive neurons have been described in other mollusks (Osborne, 1984), they had not previously been sought in either *Aplysia* or *Pleurobranchaea*.

Our results agree with those of other investigators on the distribution of immunoreactivities for CCK (Ono, 1986), FMRFamide, and SCP_B (Brown *et al.*, 1985; Lloyd *et al.*, 1987).

Evidence for diverging projection of transmitter systems in the buccal ganglia

In the buccal ganglia, immunostaining with each antiserum revealed a plexus of varicose fibers in the neuropil region. Although there were marked differences in the densities of the transmitter-specific plexuses, each plexus was observed throughout the depth of the neuropil. The diffuse, rather than regionally specific, distribution suggests that each system affects a large number of target neurons. In the case of histamine, serotonin, GABA, FMRFamide, and SCP_B, the immunostained plexus is so dense that most, or perhaps all, ganglionic neurons could be contacted by these plexuses.

On the other hand, our observations suggest that the source of each transmitter system is limited to a relatively small number of neurons. In both *Aplysia* and *Pleurobranchaea*, the potential sources of buccal neuropil include the buccal ganglion neurons themselves and those cerebral ganglion neurons that project into the buccal ganglia. Reconstruction of serial sections allowed us to estimate the number of stained neurons in both ganglia. Accordingly, the number of immunoreactive neurons in the *Aplysia* buccal ganglion was two for histamine, none for serotonin, eight for acetylcholine, less than 10 for GABA, about 10 for CCK, and about 30 for VIP. The cerebral ganglion contained 20–30 histamine-immunoreactive neurons, 20–30 serotonin-immunoreactive neurons, about 20 acetylcholine-immunoreactive neurons, 15–20 GABA-immunoreactive neurons, about 50 CCK-immunoreactive neurons, 20–30 VIP-immunoreactive neurons, and a large number of FMRFamide- and SCP_B-immunoreactive neurons. As revealed by cobalt back-injection technique, only about 40 cerebral neurons project into the buccal ganglion. In this study, however, the distribution of any immunostained set of neurons overlapped only partly with the distribution of cobalt-labeled neurons. Therefore, maybe fewer than 40 of any set of transmitter-specific cerebral neurons project into the buccal ganglion.

As for possible peripheral sources, *Aplysia* contains no ganglia between the buccal ganglion and its target tissues, and the stomatogastric ganglion of *Pleurobranchaea* is tiny and can therefore be neglected in the present discussion. In both species, some target tissues, such as the esophagus, contain a few intramural neurons. These neurons might send their axons to the buccal ganglion, but their number is small, and their possible contribution in the buccal neuropil is negligible.

Based on these estimations, we propose that, for each transmitter system, a set of relatively few neurons gives rise to a dense plexus of terminals distributed diffusely throughout the buccal neuropil. This implies that each neuron branches extensively and contacts many target neurons; *i.e.*, it shows anatomical divergence.

Cerebral neuron C1 as a source of diverging innervation

C1 is a known serotonergic neuron showing extensive divergence (Schwartz and Skolnik, 1981), so we examined how much of the serotonergic innervation in the buccal ganglion originates from this particular cell. The buccal neuropil contained an extensive serotonin-immunoreactive plexus and most buccal neurons received serotonin-immunoreactive terminals on their somas. Because the buccal ganglion contains no serotonin-immunoreactive neurons, the serotonin-immunoreactive terminals must originate in neurons outside the buccal ganglion. We did not observe any peripheral serotonin neurons, therefore we anticipated this innervation to come from the cerebral ganglion. A comparison of cerebral ganglia that had been back-injected through the cerebro-buccal connectives with ganglia that had been immunostained with serotonin antiserum and reconstructed allowed us to determine which of the cerebral serotonin-immunoreactive neurons could contribute to the buccal neuropil and, indeed, in-

dicated that the neuron C1 is the only neuron revealed by both techniques. The possibility that C1 is the sole source of buccal serotonin innervation was substantiated by the observation that serotonin antiserum stains in cross-sections of the CBC only one, large axonal profile.

Evidence for converging innervation in the buccal ganglion

All of the antisera studied stained the buccal neuropil extensively and without regional specificity. We therefore propose that any given buccal neuron is innervated by many different transmitter systems; *i.e.*, innervation from many different sources converges on the same target neurons.

Some of the substances we studied, notably the neuropeptides FMRFamide, SCP_B, VIP, and CCK, may be colocalized in the same terminals with one of the classical transmitters: histamine, acetylcholine, serotonin, or GABA (Lloyd *et al.*, 1987; Ono, 1989). Although this may be the case for selected cells, the distributions of immunostained neurons on the whole showed distinct patterns, indicating that colocalization of any two substances in molluscan ganglia is not complete. This strongly suggests that the multiple immunoreactivities reported in this study represent truly multiple innervation.

Potential models of convergence in identified neurons

Several physiologically identified neurons can be recognized in histological sections due to their size and location, including C1 of the cerebral ganglion and R2 of the abdominal ganglion. Furthermore, our preliminary studies indicate that neurons R15 and R16 are identifiable on their morphological characteristics alone. Some previously physiologically identified neurons, such as the histaminergic neuron C2, can be identified in sections due to its transmitter content (Soinila *et al.*, 1990). Our survey suggests that R15 is a potentially useful model with which to study convergence of innervation at the level of a single neuron; *i.e.*, we observed somatic connections on R15 by serotonin-, FMRFamide-, and SCP_B-immunoreactive terminals. In addition, studies by other investigators indicate that R15 is innervated by acetylcholine-, GABA-, and dopamine-containing neurons (Leake and Walker, 1980). Other candidates for multi-innervated neurons include C1, which is innervated by FMRFamide-immunoreactive terminals, as well as by histamine-immunoreactive terminals (Ono and McCaman, 1980), and C2, which receives innervation by GABA- and FMRFamide-containing terminals.

Other ganglia

The distribution of transmitter-specific systems in all ganglia had as a common feature that a relatively small number of neurons seems to give rise to an extensive plexus of fibers. These plexuses (with the exception of GABA- and acetylcholine-immunoreactive ones in the cerebral ganglion) do not show regional specificity in their projection. Although our evidence on divergence and convergence of innervation was obtained mainly in the buccal ganglion, anatomical similarities in distribution patterns suggest that these properties will be common for other ganglia as well.

Conclusions

In the present study, divergence and convergence were anatomically demonstrated in individual identifiable neurons. For whole ganglia, the evidence for divergence is indirect and comes from the observation that the number of contributing neurons is small in comparison to the extent of contacts each system seems to make. Because our material consisted of complete serial sections, the number of immunoreactive neurons can be reliably estimated. We stipulate that additional neurons may contain these substances in levels too low to be detected immunohistochemically. In some systems, evidence has been reported that transmitter or neuropeptide concentration in the neuronal soma is considerably lower than that in the corresponding terminals (Storm-Mathiessen and Ottersen, 1986). However, our assumption on divergence seems reasonable because the stained neurons, except for FMRFamide-immunoreactive ones, represent only a fraction of the total ganglionic neuron population.

Our conclusion on divergence is based on two assumptions: (1) that the density of varicose terminals correlates with the extent of innervation; and (2) that lack of regional specificity in the distribution of terminals indicates that most, if not all, ganglionic neurons are targets of that particular system. The first assumption can be made because, in a wide variety of species, chemical synapses are located in terminal boutons that are visualized as varicosities at the light microscopic level. In the present material, the number of fibers is clearly correlated with the number of varicosities. The second assumption rests on the argument that, if diffusely distributed terminals contact only specific target neurons, there should be a precise recognition mechanism guiding the right innervation to the right targets during the ontogeny. Also, in such systems, the circuits must be strictly isolated electrically and protected against the diffusion of transmitters from nearby terminals. The ultrastructure of the molluscan neuropil is incompletely described, so such a possibility cannot be excluded.

Our conclusion about the convergence of innervation in whole ganglia is based on the deduction that the more extensively several systems diverge, the more probable that any given neuron receives multiple innervation. Al-

though we have presented evidence that neurons receive innervation by several transmitter systems, we cannot be sure that all these contacts are functional. Previous studies suggest, however, that electrophysiological convergence and divergence occur commonly in the nervous systems of both *Aplysia* (Kandel and Tauc, 1968; Kandel and Wachtel, 1975; Shimara and Tauc, 1975) and *Pleurobranchaea* (Mpitsos and Cohan, 1986b). In future studies, transmitter histochemistry should be accompanied by corresponding receptor mapping, to determine whether a particular transmitter-specific neuron would be capable of responding to multiple inputs, and further by electrophysiological demonstration of the corresponding response. While selective projections might occur in extensively overlapping fields, as shown, for example, in Figure 2, we hypothesize that the tangle of fibers converging to the same area may not be motor specific. Thus, whereas specific, distributed convergence poses difficulties in genetic encoding or developmental expression, the possibility of nonspecific projection poses difficulties for demonstrating how behavior specific activity emerges appropriately.

Broader implications

The findings presented here are consistent with our previous studies indicating that neural systems, just as other adaptive systems, self-organize their activity by distributed, often error-prone action (Mpitsos and Cohan, 1986a, b; Burton and Mpitsos, 1991). "Neurocircuits" and the units that comprise them may have "fuzzy" functional characteristics (Zedah, 1965; Mpitsos, 1989) rather than exact, reliably identifiable ones. To be sure, one cannot deny the extensive evidence showing that relatively fixed action does occur, although we also believe that many instances of variability have been overlooked. From a broader analogy, as Newtonian physics has difficulty in growing into statistical mechanics, so classical definitions of response-specific neurocircuits have difficulty in accounting for flexible neural function. Nonetheless, theories that account for flexible action must also account for instances in which flexibility is not observed. This is to say that a general solution to the problem must also provide evidence for the more special case.

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